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Presence of starch enhances *in vitro* biodegradation and biocompatibility of a gentamicin delivery formulation

Elizabeth R. Balmayor,[1,2]* Turker E. Baran,[1,2] Marina Unger,[3] Alexandra P. Marques,[1,2] Helena S. Azevedo,[1,2] Rui L. Reis[1,2]

[1]3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal
[2]IBB – Institute for Biotechnology and Bioengineering, PT Associated Laboratory, AvePark, 4806-909 Taipas, Guimarães, Portugal
[3]Department of Experimental Trauma Surgery, Klinikum rechts der Isar, Technical University Munich, Ismaninger Strasse 22, D-81675 Munich, Germany

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Abstract: The effect of α-amylase degradation on the release of gentamicin from starch-conjugated chitosan microparticles was investigated up to 60 days. Scanning electron microscopic observations showed an increase in the porosity and surface roughness of the microparticles as well as reduced diameters. This was confirmed by 67% weight loss of the microparticles in the presence of α-amylase. Over time, a highly porous matrix was obtained leading to increased permeability and increased water uptake with possible diffusion of gentamicin. Indeed, a faster release of gentamicin was observed with α-amylase. Starch-conjugated chitosan particles are non-toxic and highly biocompatible for an osteoblast (SaOs-2) and fibroblast (L929) cell line as well as adipose-derived stem cells. When differently produced starch-conjugated chitosan particles were tested, their cytotoxic effect on SaOs-2 cells was found to be dependent on the crosslinking agent and on the amount of starch used. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 00B: 000–000, 2014.

Key Words: starch-conjugated chitosan, gentamicin, microparticles, enzymatic degradation, cytotoxicity

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INTRODUCTION

Biomaterials with the ability to perform certain medical functions and subsequently degrade are of great importance. Their degradation should occur under a controlled mechanism and into easily metabolized products.[1,2] Among the well-known advantages of using biodegradable materials are the elimination of the long-term presence of foreign materials and the consequent need for a second surgery for implant removal.[1]

Several definitions for biodegradability have been proposed.[1,3,4] In the context of biomedical applications, biodegradation may be defined as the "gradual breakdown of a material mediated by a specific biological activity."[4] When in contact with tissues, biomaterials start to degrade as a result of their interaction with biological environments. Their stability will define their application in clinics. In drug delivery applications, biodegradability of the carrier system affects and finally controls the release kinetics of the encapsulated drugs.[5] Thus, the polymeric carriers should degrade under physiological conditions allowing for the gradual release of the entrapped drug as well as facilitate repeating dosages to ensure successful treatments.[6,7] At this point, a major concern related to biodegradable polymers is the possible toxicity of the leachable molecules and degradation products derived from the material processing and degradation.[8] Thus, the nature of the materials to be selected as drug delivery carriers is also a rather important factor to consider. Polysaccharides are very attractive in this respect as the degradation process results in nontoxic end products such as glucose, maltose, and oligosaccharides. These end products may even be incorporated in the normal metabolic pathways of the human body.[1,9]

Chitosan and starch have been extensively employed as a carrier in drug delivery systems.[9–13] Both natural polymers are biocompatible and biodegradable.[9] In particular, starch is a cheap, abundant and edible polysaccharide with

Correspondence to: E. R. Balmayor; e-mail: elizabeth.rosado-balmayor@tum.de
**Present address:* Clinic for Plastic Surgery and Hand Surgery, Technical University Munich, Ismaninger Strasse 22, 81675 Munich, Germany
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interesting biodegradable properties.[1] Our group has dedicated more than a decade to the research of this polymer.[11,14–17] Thus, based on our findings, we hypothesize that starch—when blended with chitosan—could improve the processing characteristics as well as the degradation and biocompatibility of the chitosan formulation alone.

We previously developed starch-chitosan (ST-CHT) microparticles formulation loaded with gentamicin sulfate (GTM) and demonstrated the capacity of the system to provide a long-term sustained release of biologically active GTM.[11] Our intention was to develop a suitable system to treat bone infections like osteomyelitis. GTM is an antibiotic characterized by a wide antibacterial spectrum.[18] It is known to be very efficient in treatments of complicated infections caused by gram-positive bacteria such as *Staphylococcus aureus*.[18] This bacterium is commonly the cause of osteomyelitis. Thus, GTM has been the antibiotic of choice to deal with this type of infection for decades.[19] Therefore, we choose GTM to be incorporated in our system.

Here, we investigated the biodegradability and biocompatibility of the GTM-loaded microparticles. Particular interest was centered on enzymatic degradation by α-amylase. This enzyme is present in many tissues in the human body including blood[1] and saliva.[20] In addition, it has a specific action in cleaving the α (1–4) glycosidic bond of starch molecules. Thus, it is expected that α-amylase participates in the degradation of starch-based biomaterials when placed in contact with human fluids, tissues and organs. Moreover, the effect of α-amylase degradation on the release kinetics of entrapped GTM from the ST-CHT microparticles was also studied. The extent of degradation was evaluated by measuring the weight loss and the presence of reducing sugars in the degradation medium. Changes in the chemical structure as well as in their morphological features were also analyzed. The biocompatibility of the developed microparticles was assessed by the use of an osteoblast and a fibroblast cell line as well as human primary stem cells. The effect of material processing conditions such as crosslinking agent, particle size, particle concentration, and amount of starch were thoroughly evaluated by using an osteoblast cell line.

MATERIALS AND METHODS

Materials

The materials studied in this work were chitosan (the viscosity average molecular weight was found to be 385 kDa and the deacetylation degree 81.25%)[11] from Sigma-Aldrich (USA) and soluble potato starch supplied by ATO (The Netherlands). Both materials were used as received. α-amylase (EC 3.2.1.1) from *Aspergillus Oryzae*, 215 U/mg protein, was obtained from Sigma-Aldrich. Other chemicals were analytical grade, from Sigma-Aldrich and were used as received.

The human osteoblast cell line (SaOs-2) and the mouse fibroblast cell line (L929) were purchased from the European Collection of Cell Cultures (ECACC, UK). The human adipose-derived stem cells were kindly provided by Professor Martijn van Griensven, MD, PhD (Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria).

Preparation of starch-conjugated chitosan microparticles loaded with gentamicin

ST-CHT conjugate was prepared by an initial reductive alkylation crosslinking method.[21] Briefly, starch was oxidized to produce a polyaldehyde that reacts with the amino group of chitosan in the presence of a reducing agent. The production of the ST-CHT microparticles was then carried out using a water in oil emulsion technique, as described previously.[11] In brief, equal volumes of chitosan (1.5%, w/v) and oxidized starch (2%, w/v) solutions were mixed, and the pH adjusted to 5.5 by using 0.5 M NaOH. The polymeric solution was added dropwise into a glass reactor containing mineral oil with 1% of Tween 80 and emulsified with a top stirrer at 600 rpm for 30 min. After emulsion formation, 30 mL of cold acetone was added slowly. The microparticles were then removed from the reaction medium by filtration and washed with distilled water/acetone mixture (1:1 v/v). After alkaline (sodium carbonate buffer solution, 0.5*M*) and reduction (sodium borohydride solution, 0.05%) treatments, the microparticles were washed with distilled water and allowed to dry at room temperature.

For the particles to be loaded with GTM, 15 mg of GTM per mL polymer solution was directly added to the polymeric solution before the particles formation. Subsequently, the microparticles were formed following the protocol described above.

For sterilization of the microparticles, ethylene oxide (EtO) was used. To perform this, two consecutive EtO cycles (of ~25 hs each) were applied in an industrial autoclave at 45°C, in an atmosphere with 88% CO_2, 12% EtO, and 50% humidity. These sterilization conditions have been previously optimized for starch and starch blended materials with satisfactory results.[22]

Enzymatic degradation study: *In vitro* release of gentamicin in an enzymatic environment

Pre-weighed samples (unloaded and GTM-loaded ST-CHT microparticles listed in Table I) were incubated in a 1:10 ratio in 0.01*M* phosphate buffered saline solution (PBS, pH = 7.4) containing α-amylase 150.5 U/L, at 37°C and under constant shaking (50 rpm) up to 8 weeks. The concentration of α-amylase used in the degradation solution is in the range of the physiological concentration of this enzyme found in human blood.[23] The buffered enzyme solution was changed weekly to maintain the desired level of enzyme activity throughout the experiment. For the preservation of the degradation solution, sodium azide (0.02%) was added to the buffered enzyme solution (stored at 4°C for further use). As a control, the samples were incubated in the presence of PBS only. At the end of each degradation time (1, 2, 3, 7, 14, 30, and 60 days), the supernatants were removed and stored at −80°C for further analysis. The excess of solution was carefully removed with filter paper and the samples were weighed using an analytical balance (±0.01 mg accuracy) for the determination of water

TABLE I. Starch-Conjugated Chitosan Microparticle Formulations used in the Enzymatic Degradation Study

Sample[a]	GTM (mg/mL)	Starch (%)	Stirring Rate (rpm)	Size (μm)	EE (%)
ST-CHT 1a	–	2	600	96.1 ± 12.4	–
ST-CHT/G 1	15	2	600	116.6 ± 31.8	55.01±2.65

Unloaded and gentamicin loaded microparticles were incubated in the presence of α-amylase at physiological concentrations up to 8 weeks. The *in vitro* release of gentamicin was also evaluated in this enzymatic environment.
ST-CHT: starch-conjugated chitosan microparticles.
ST-CHT/G: gentamicin loaded starch-conjugated chitosan microparticles.
[a] Microparticles obtained using standard experimental conditions as described in the Materials and Methods section and elsewhere.[11]

uptake.[8] Subsequently, the samples were thoroughly washed with distilled water and allowed to dry at room temperature until presenting constant weight. The final weight of the samples was used for the calculation of the weight loss in relation to the initial weight.[8] The supernatants from the degradation solutions were used to quantify the amount of released GTM and to determine the concentration of reducing sugars released into the solution as a product of the degradation process.

The quantification of the released GTM was performed by an indirect spectrophotometric method using o-phtaldialdehyde as derivatizing agent.[11] Further details on this method can be found elsewhere.[11,24] For the spectrophotometric determination, equal volumes of GTM-containing solutions, *o*-Phtaldialdehyde reagent and isopropanol were mixed. After 30 min of incubation at room temperature, the absorbance was measured at 332 nm. The GTM amounts were calculated according to a standard curve. The samples were tested in triplicates in three independent assays.

The amount of reducing sugars in the supernatants was estimated by the dinitrosalicylic acid (DNS) method.[25] A calibration curve was prepared using glucose solutions of known concentrations. Briefly, standard solutions and samples were mixed with the DNS reagent (mixture of DNS and Rochelle salt) under the appropriate conditions and allowed to react at 100°C for 5 min. Finally, the absorbance was determined using a microplate reader at 540 nm (EL 312e Biokinetics, BioTek Instruments). The amount of reducing sugars was reported as the average concentration of three replicate determinations.

Chemical and physical characterization

Morphological evaluation: Scanning electron microscopy (SEM). SEM was used to assess the changes on the microparticles' morphology as result of the degradation process. Following a standard procedure for sample preparation, the microparticles were mounted onto metal stubs by using a carbon tape and gold sputter-coated (Sputter Coater SC502, Fison Instruments, UK). Samples were analysed by SEM (Leica Cambridge S-360 model, UK) after the different points in time of degradation studied.

Chemical changes: FTIR. To assess possible changes in the chemical composition of the microparticles as result of the enzymatic degradation, the samples were analyzed by FTIR spectroscopy in a transmission mode (IRPrestige-21 FTIR-8400S, Shimadzu, Japan) before and after the degradation process. For the preparation of the samples for FTIR analysis, 1 mg of sample was mixed with 40 mg of spectroscopic KBr, and then processed into a disc using a manual press (161–1100 hand press, PIKE Technologies, USA). Transmission spectra were recorded using at least 32 scans with 4 cm^{-1} resolution and in the spectral range 4000–600 cm^{-1}.

Nuclear magnetic resonance spectroscopy (^{1}H-NMR). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained (Varian Unit Plus 300 MHz spectrometer) under the following experimental parameters: 256 scans, 5200 Hz filter width, 90° pulse corresponding to a pulse width of 11 μs and 4 s relaxation delay. The experiments were run at 60°C. For the sample preparation, 10 mg of each sample were dissolved into a mixture of deuterated solvents, 0.98 mL of deuterium oxide (D_2O) and 0.02 mL of deuterium chloride until complete dissolution and transferred into a NMR tube ($\varphi = 0.3$ mm).

Evaluation of cytotoxicity

The biocompatibility of the ST-CHT microparticles was assessed by performing a standard MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2–(4-sulfophenyl)-2H-tetrazolium) assay. A human osteoblast cell line (SaOs-2), a mouse fibroblast cell line (L929) and human adipose-derived mesenchymal stem cells (AMSCs) were used for this purpose. The microparticles were placed in direct contact with cells to evaluate their effect on cell viability. Thus, SaOs-2, L929 and AMSCs were cultured in 12-well tissue culture plates at a density of 1×10^5 cells/mL having 1.5 mL/well, using Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% of heat-inactivated fetal bovine serum (FBS, Biochrom AG, Germany) and 1% of antibiotic/antimycotic solution (Sigma-Aldrich). Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% of CO_2. At that point in time, the culture medium was replaced by a suspension of the ST-CHT microparticles (previously sterilized by EtO) in DMEM complete medium (1 mg/mL). After 72 h incubation time, the particles were removed by aspiration to perform the cytotoxicity assay. The MTS test was performed according to the manufacturer's instructions provided in the CellTiter 96 One Solution Proliferation Assay Kit (Promega). Briefly, after the incubation period, the cell monolayer was treated with 200 μL/well of MTS reagent solution (5:1 ratio in serum-

TABLE II. Starch-Conjugated Chitosan Microparticles used for the Biocompatibility Evaluation

Sample	GA (%)	TPP (%)	Starch (%)	Stirring Rate (rpm)	Size (μm)	Cell Viability (% Control)
ST-CHT 1[a]	–	–	2	600	96.1 ± 12.4	77.12 ± 6.2
ST-CHT 2	–	–	1	600	87.7 ± 29.1	71.18 ± 9.1
ST-CHT 3	–	–	4	600	91.3 ± 9.8	91.24 ± 1.8
ST-CHT 4	0.5	–	2	600	79.6 ± 6.3	73.21 ± 8.6
ST-CHT 5	1	–	2	600	75.3 ± 13.4	65.83 ± 5.9
ST-CHT 6	2	–	2	600	74.3 ± 11.7	55.87 ± 9.1
ST-CHT 7	–	0.5	2	600	82.9 ± 9.5	79.81 ± 4.0
ST-CHT 8	–	1	2	600	70.4 ± 10.4	76.78 ± 7.2
ST-CHT 9	–	2	2	600	71.3 ± 24.8	78.81 ± 3.8
ST-CHT 10	–	–	2	400	207.1 ± 11.6	74.70 ± 8.9
ST-CHT 11	–	–	2	800	60 ± 7.4	76.27 ± 6.6
ST-CHT 12	–	–	2	1000	10 ± 9.1	79.30 ± 2.4

Twelve different microparticles formulations were obtained by modifying the standard processing conditions. The effect of the modifications on the resulted cytotoxicity is expressed as percentage of cell viability in comparison to control (100% viability)
Common experimental conditions for all experiments:
Volume of polymer solution: 10 mL.
Mineral Oil 70 mL [1% Tween 80, (v/v)].
GA: glutaraldehyde; ST-CHT: starch-conjugated chitosan microparticles; TPP: sodium tripolyphosphate
Percentages of viable cell compared to negative control (100%-DMEM culture medium) as a result of the direct contact with starch-conjugated chitosan microparticles
[a] Standard processing conditions as described in the Materials and Methods section and elsewhere.[11]

free DMEM culture medium without phenol red) and incubated for further 3 h at 37°C and in a humidified environment containing 5% of CO_2; 100 μL of each well were then filtered (0.45 μm pore size) to eliminate the possible presence of solid particles, and transferred to a new 96-well plate. The absorbance was determined in a microplate reader (EL 312e Biokinetics, BioTek Instruments) at 490 nm. The results are expressed as the percentage of cell viability in comparison with the negative control (i.e., for each cell type, cells cultured in DMEM complete medium in the absence of microparticles) for which 100% viability was attributed. Latex rubber extract was used as positive control (cytotoxic effect).[8] The samples were tested in triplicates in three independent assays.

A further aim of our study was to evaluate the influence of several variations on the experimental conditions used to produce different types of ST-CHT microparticles regarding the particles' biocompatibility. Thus, three modifications were introduced to the standard procedure previously described to produce the particles[11]: (i) two different crosslinking agents, sodium tripolyphosphate (TPP), and glutaraldehyde (GA), at different concentrations (0.5, 1, and 2%) were employed. Crosslinking was achieved by immersing the obtained samples for 3 h into the crosslinker agent solution, (ii) the amount of starch in the composition of the ST-CHT conjugate polymer solution used for the microparticles preparation was varied (1, 2, and 4%), iii) the stirring rate employed to form the emulsion was increased from 400 to 1000 rpm aiming at producing smaller sizes microparticles. By means of the above mentioned modifications, 12 new ST-CHT microparticles formulations were obtained at this point. Table II shows the experimental conditions used to produce the new different types of ST-CHT microparticles to test them in the cytotoxicity screening. In addition, different particle concentrations were also tested. Moreover, the cytotoxicity resulting from the contact of the material extracts over the cells was compared to the direct contact particles-cell monolayer.

To obtain the ST-CHT microparticles extracts, the particles (formulation ST-CHT 1, Table II) were incubated in complete cell culture medium for 24, 48, and 72 h at 37°C under constant shaking (100 rpm). This incubation was performed using 0.5 mg microparticles per mL of complete culture medium. Subsequently to the incubation period, the extraction medium containing potential toxic leachable and degradation products was filtered (0.45 μm pore size) and added to the 80% confluence cell layer. The cells were incubated for further 72 h with this medium at 37°C and in a humidified environment containing 5% of CO_2. The MTS assay was performed for all microparticles tested, as well as the particle extracts, as described above.

For all these cytotoxicity screening processes, the human osteoblast cell line SaOs-2 was selected as cell type. In addition to the cytotoxicity screening using the unloaded microparticles, GTM-loaded particles were also tested (formulation ST-CHT/G 1, Table I). Thus, SaOs-2 and L929 cell lines and AMSCs were used. Fluorescence microscopy pictures (Biorevo BZ9000, Keyence, Japan) were taken 24 and 72 h after culture of the GTM-loaded particles with the cells. Calcein-AM and propidium iodide was used for a live/death staining.

Statistical analysis

All obtained values are reported as mean ± standard deviation. The statistical analysis was performed with OriginPro 8.0 (Microcal1 software; OriginLab Corp). Normal distribution of the data was analyzed by Shapiro-Wilk test. Differences between samples were determined using Student's *t test* for two independent samples. p values < 0.05 were considered significant.

RESULTS

Enzymatic degradation

Physicochemical characterization: Water uptake and weight loss. As a result of immersion in aqueous solution, hydrophilic materials undergo a process of swelling with posterior uptake of large amounts of fluid. Figure 1(A) shows the water uptake percentage of the ST-CHT microparticles over a period of 60 days of incubation. In comparison to PBS, significantly higher values of water uptake (160.1 ± 5.6)% were obtained after incubation of the particles in α-amylase solution ($p = 0.004$). α-amylase was used at a physiological concentration throughout the study.

The weight loss of ST-CHT microparticles is represented graphically in Figure 1(B). Higher weight loss, that is, a higher degradation rate, was obtained for the microparticles incubated in the presence of α-amylase in comparison to plain PBS ($p = 0.00003$). In fact, after 60 days of immersion in the α-amylase solution, the ST-CHT microparticles were extensively degraded, characterized by 67% of weight loss. In contrast, when the particles were incubated in plain PBS only 3% of weight loss was recorded. An association between the weight loss and the extent of the particles' degradation can be concluded from the inserted SEM micrograph in Figure 1(B). The micrographs clearly demonstrate the loss of the material integrity concomitant with the weight loss.

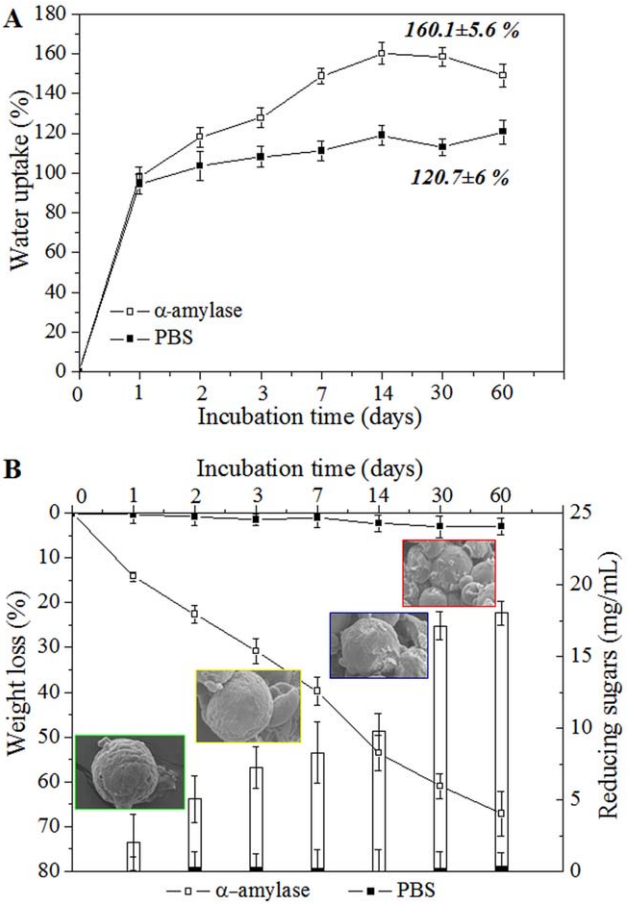

FIGURE 1. A: Water uptake profile of starch-conjugated chitosan microparticles in PBS and PBS containing α-amylase (37 °C, 100 rpm). B: Degradation profiles (expressed as weight loss percentage) and concentration of reducing sugars released from the microparticles into the solution after incubation in PBS, and PBS containing α-amylase (37 °C, 100 rpm). Line graphs correspond to weight loss and bar graphs represent the concentration of reducing sugars. The data points in the figure represent the mean of three replicates ($n = 3$) and the error bars the standard deviation. Insert SEM micrographs shows the extent of the material degradation concomitant with the degradation profiles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Determination of reducing sugars in the supernatants. It is well-known that enzymatic hydrolysis of polysaccharides is accompanied by the release of glucose and several other soluble sugar molecules to the degradation media.[1] Figure 1(B) (bars) shows the concentration of reducing sugars released into the solution after incubation of the ST-CHT microparticles with α-amylase. Reducing sugars were found in the supernatants where the microparticles were incubated in the presence of the enzyme, but not in the control supernatants (PBS only). As expected, prolongation of the incubation time led to significantly increasing amounts of reducing sugars ($p = 0.0002$). In fact, after 60 days of degradation, the concentration of reducing sugars quantified was (18.02 ± 0.86) mg/mL.

Morphological evaluation of the degradation process: SEM. The SEM micrographs presented in Figure 2 show the morphology of ST-CHT microparticles before and after incubation with PBS and with the enzymatic degradation solution. In the presence of the enzyme, the microparticles' size decreased and fractures and pores appeared at the surface [Figure 2(E–H)]. In addition, the particles lost their spherical shape, indicating the progression of the degradation of the particles when placed in contact with α-amylase in contrast to PBS [Figure 2(C,D)].

Chemical evaluation of the degradation process: FTIR-Nuclear magnetic resonance (^{1}H-NMR). Figure 3(A) shows the FTIR spectra obtained for ST-CHT microparticles before and after 60 days of α-amylase degradation. Relevant bands assigned to polysaccharides as well as chitosan and starch can be clearly identified. In the range of 900–1200 cm^{-1}, the carbohydrate fingerprint region, the three major starch-specific bands 1030, 1080, and 1150 cm^{-1} for the C—O—C stretching can be clearly identified (triplet, broad band). After the enzymatic hydrolysis, a clear decrease in the intensity of those bands can be observed, indicating the action of α-amylase in cleaving the C—O—C glycosidic linkages of starch. On the contrary, when ST-CHT microparticles were incubated in PBS only, no gross changes in chemical composition were observed.

Figure 3(B) shows the ^{1}H-NMR spectrum of ST-CHT microparticles before and after α-amylase degradation. The spectrum of chitosan raw material is presented for comparison. After 60 days of α-amylase degradation, this peak at 5.5 ppm disappears [Figure 3(B)]. This peak corresponds to the anomeric proton associated with α (1→4) linkages of

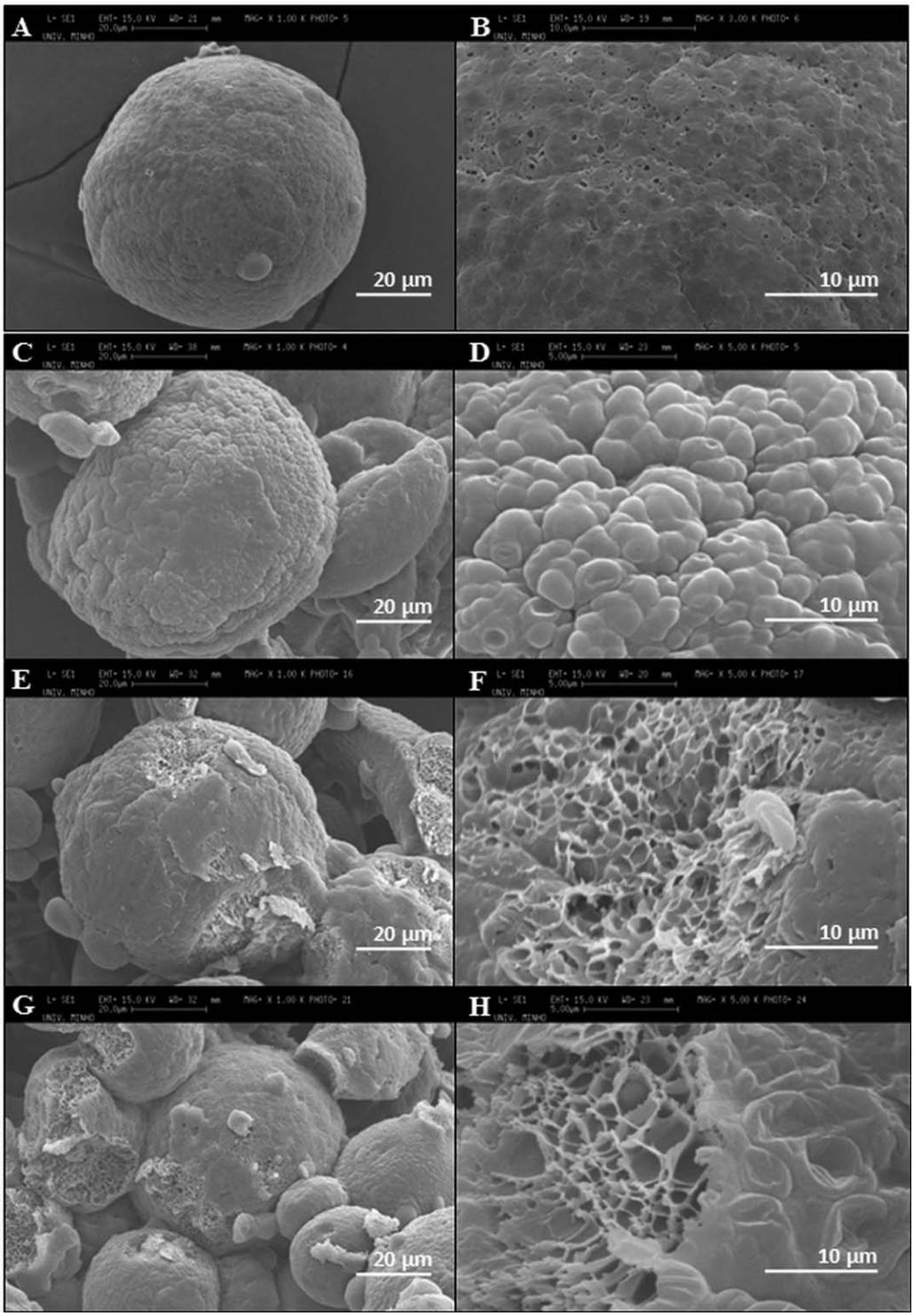

FIGURE 2. SEM micrographs showing the morphology of surfaces and the shape of starch-conjugated chitosan microparticles. A,B: before degradation. C,D: 60 days after incubation in plain PBS solution. E,F: 14 days after incubation in α-amylase solution. G,H: 60 days after incubation in α-amylase solution.

the starch material. Thus, this is an indication of the action of the enzyme cleaving the glycosidic linkages in the starch. In addition, the multiplet signal at 4-ppm, assigned to the internal protons of starch, also disappear after α-amylase degradation. As a result, changes in the multiplet signal shape can be observed, leading to the clarification of the chitosan-characteristic peaks that were overlapped with the starch multiplets in the same region of the spectrum.

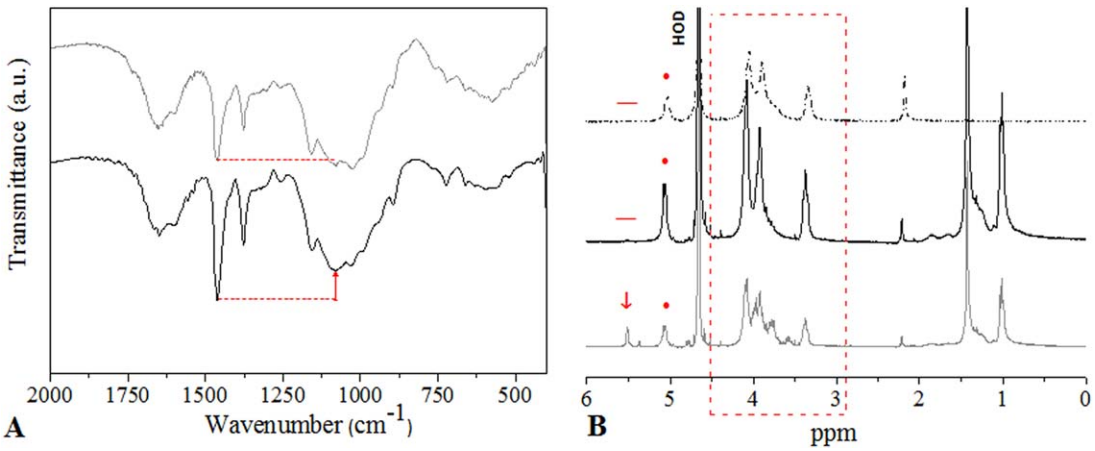

FIGURE 3. A: FTIR spectra of starch-conjugated chitosan microparticles before (gray) and after (black) enzymatic degradation with α-amylase for 60 days. The diminution in the intensity of the bands, as result of the action of α-amylase cleaving the C—O—C glycosidic linkages of starch, is indicated with an arrow. B: ^{1}H-NMR spectra of the microparticles before (gray) and after (black) 60 days of degradation in presence of α-amylase. The spectrum of chitosan raw material used to produce the microparticles is presented for comparison (discontinuous line). Symbols indicating peaks or group of peaks: ↓: starch (α (1→4) glycosidic linkages, singlet), •: chitosan (deacetylated unit, doublet), —: area- starch (internal protons, multiplets). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of α-amylase degradation on the release kinetics of gentamicin

Figure 4 shows the release profiles of GTM from ST-CHT microparticles in PBS and in the presence of α-amylase. Interestingly, the initial burst release (up to 12 h) of the GTM was unaffected by the presence of the enzyme. However, from that point in time on, the GTM was released significantly faster when α-amylase was present ($p = 0.028$). This effect became more pronounced from the second day on, where 71% GTM was released from the ST-CHT microparticles in the presence of the enzyme, whereas only 59% was released from the sample incubated in PBS only.

Cytotoxicity evaluation

The biocompatibility of the ST-CHT microparticles was evaluated by means of a cytotoxicity screening using several cell types. Both unloaded and GTM-loaded microparticles were evaluated. The microparticles were found to be highly biocompatible with SaOs-2 (osteoblasts) and L929 (fibroblasts) cells [Figure 5(A)]; 77.12% cell viability was obtained as a result of incubating the unloaded particles with osteoblast cells SaOs-2 for 72 h. Similar cell viability values, that is, 70.01%, were obtained for the GTM-loaded microparticles. In fact, no significant difference was observed on the biocompatibility of unloaded and GTM-loaded microparticles over SaOs-2. The cells appeared microscopically healthy and no visual differences could be observed when comparing with the control cells (cultured without microparticles). For the L929 cell line, 83.08% viability was obtained for the unloaded particles whereas 89.72% viability was obtained for the GTM-loaded particles after 72 h incubation. The particles added to the fibroblast culture did not result into any detrimental effect. No significant differences were obtained for the cell viability values when comparing them to the negative control ($p = 0.064$). However, cell viability values higher than 76% were obtained as a result of incubating the microparticles for 72 h with AMSCs. No significant difference was observed on the biocompatibility of unloaded and GTM-loaded microparticles over AMSCs. The calcein-AM/propidium iodide results [Figure 5(B–G)] are in accordance with the MTS findings. All cells appear to be viable (green fluoresce indicating viable cells) while very few cells seem to be nonviable (red fluoresce indicating apoptotic cells).

Effect of material extracts. A further cytotoxicity evaluation was conducted using extracts of the ST-CHT microparticles over SaOs-2 cells. Figure 6(A) shows that the extraction time does not significantly influence cell viability ($p = 0.2$). Cell viability values were found to be above 70%, when compared with negative control (100%).

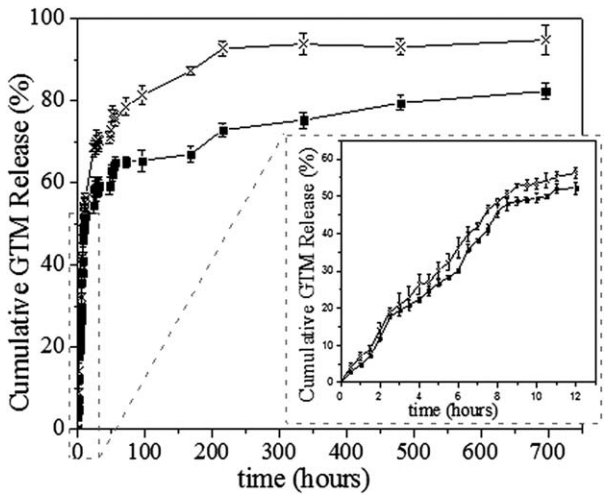

FIGURE 4. Release profiles of gentamicin from starch-conjugated chitosan microparticles in PBS (0.01*M*, pH = 7.4) **(-■-)** and PBS containing 150.0 U/L α-amylase **(-x-)** solutions.

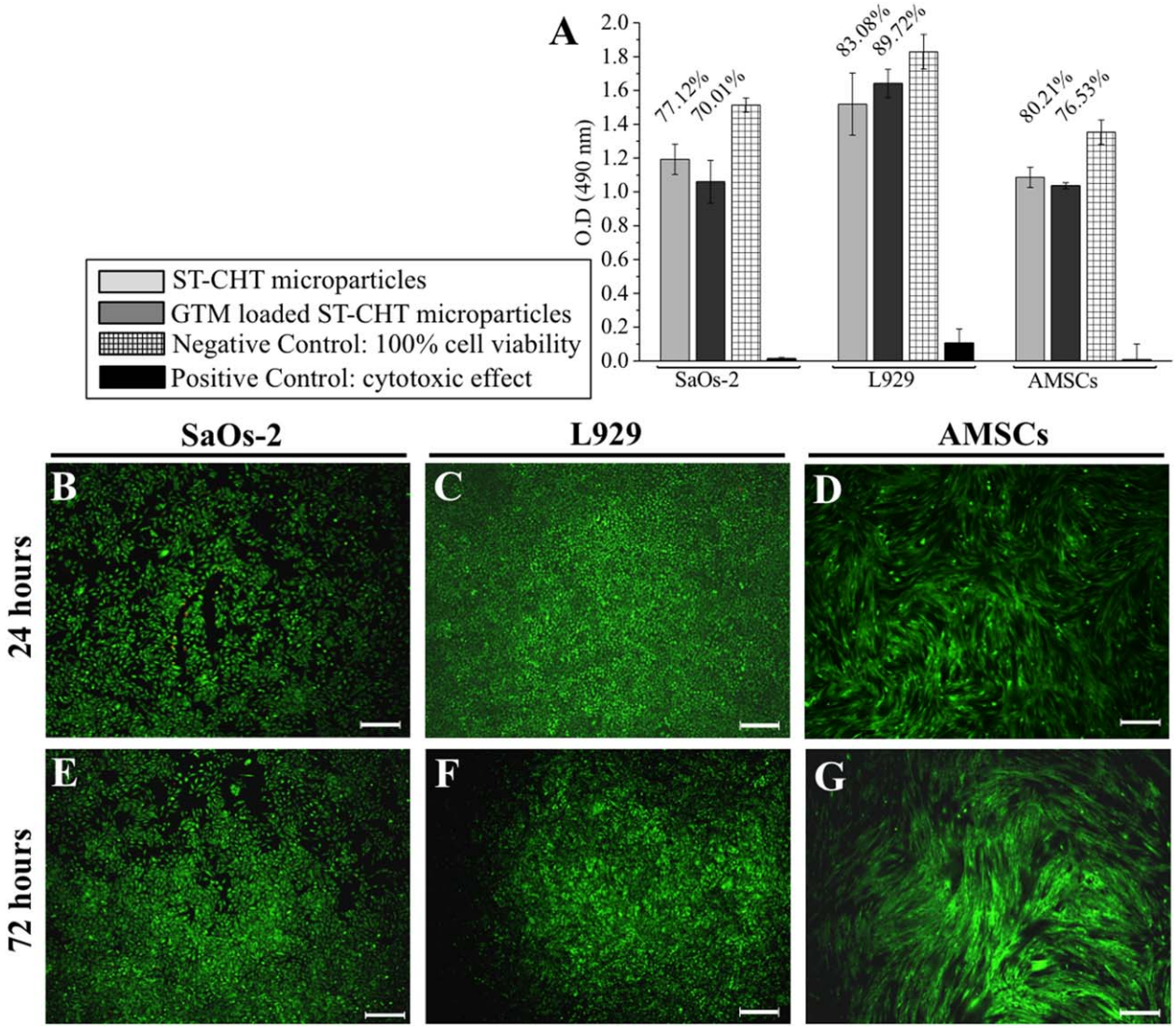

FIGURE 5. Biocompatibility screening of the starch-conjugated chitosan microparticles by means of MTS assay. A: Optical density of the MTS solutions after culture of SaOs2, L929, and AMSCs in the presence of unloaded and gentamicin-loaded starch-conjugated chitosan microparticles for 72 h (microparticles ST-CHT 1 and ST-CHT/G 1 from Table I were used). Calcein-AM and propidium iodide (live/death staining) pictures of SaOs-2, L929, and AMSCs after culture in the presence of gentamicin-loaded starch-conjugated chitosan microparticles for: (B–D) 24 h and (E–G) 72 h. The scale bars represent 400 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Effect of the crosslinking agent and its concentration. Two different crosslinking agents, that is, GA and TPP at three different concentrations were used to provide structural stability to the microparticles. Table II summarizes the percentage of cell viability depending on the concentration and on the crosslinking agent used. Significantly higher cytotoxicity (55.87% cell viability) was obtained for GA when compared with TPP ($p = 0.002$) and to the negative control (100%; $p = 0.0002$). This toxic effect was found to be concentration-dependent. A considerable improvement in cell viability (i.e., over 70%) was observed when decreasing the GA concentration from 2 to 0.5% [Table II and Figure 6(B)]. However, values higher than 75% were obtained for cell viability when TPP is used as a crosslinking agent. Those resulted independently of the concentration used. In fact, the cell viability results are in the same range as the ones obtained when no crosslinking agent was used ($p = 0.66$).[11]

Effect of the amount of starch in the composition of the ST-CHT microparticles. An enhancement of cell viability was obtained by increasing the amount of starch in the composition of the ST-CHT microparticles [Figure 6(C)]. Cell viability values increased from 71 to 91% when the concentration of starch was raised from 1 to 4% (Table II).

Effect of the ST-CHT microparticles' concentration. A range from 0.25 to 5 mg particles per mL cell culture medium was selected to test the effect of the microparticles' concentration. Cell viability values >70% were obtained for each concentration [Figure 6(D)]. No significant differences

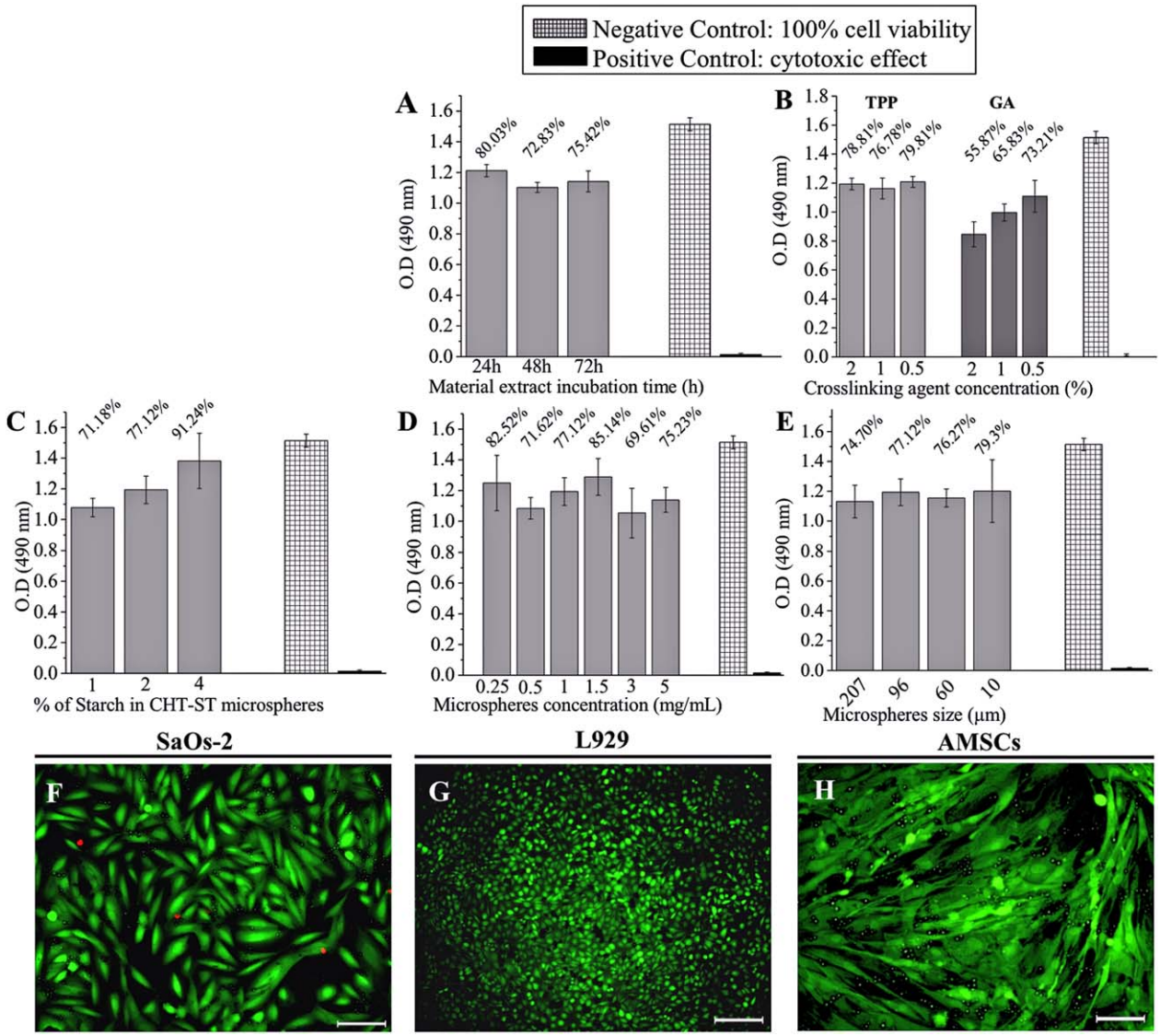

FIGURE 6. Biocompatibility screening of the starch-conjugated chitosan microparticles produced by variations on the processing parameters. Optical density of the MTS solutions after culture of SaOs2 for 72 h in the presence of: A: microparticles extracts obtained in culture medium for 24, 48, and 72 h (microparticles ST-CHT 1 from Table II were used), B: microparticles obtained by the use of different concentrations of GA and tripolyphosphate as crosslinking agents (microparticles ST-CHT 4–9 from Table II were used), C: microparticles obtained by the use of different percentages of starch in their composition (microparticles ST-CHT 1–3 from Table II were used), D: different concentrations of microparticles in the range of 0.25–5 mg/mL culture medium (microparticles ST-CHT 1 from Table II were used), E: microparticles with different sizes (microparticles ST-CHT 1, 10–12 from Table II were used). Calcein-AM and propidium iodide (live/death staining) pictures of the cells cultured in direct contact with the starch-conjugated chitosan microparticles: (F) SaOs-2, (G) L929, and (H) AMSCs. The scale bars represent 200 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

resulted from the comparison among the different microparticles' concentrations tested.

Effect of the ST-CHT microparticles' size. By increasing the stirring speed from 400 to 1000 rpm during the microparticles' production, particles with sizes from 207.1 ± 11.6 to 10 ± 9.1 μm were obtained. Decreasing the microparticles' size did not induce a significant difference in cell viability [Figure 6(E)]. Values of cell viability higher than 74% were obtained for all four differently sized particles tested. No significant differences were obtained when the particles were compared among them. Figure 6(F–H) shows the results of the calcein-AM/propidium iodide staining for SaOs-2, L929, and AMSCs cultured in direct contact with the smallest ST-CHT microparticles obtained in our study (size 10 ± 9.1 μm). The cells appear to be highly viable as indicated by the markedly green fluorescence patterns. No cell death was observed.

DISCUSSION

A biomaterial designed for biomedical applications will interact with human tissues and fluids after implantation, which might initiate their degradation process. Chemical and enzymatic oxidations, nonenzymatic hydrolysis

(catalyzed by acids, bases, or salts) and enzymatic hydrolysis caused by the action of enzymes are some of the routes for degradation to occur.[1] α-amylase is expected to participate in the degradation of starch-based biomaterials that perform their function within the human body (i.e., in those tissues were this enzyme occurs naturally). Here, we show that the developed ST-CHT microparticles were susceptible to degradation by physiological concentrations of α-amylase. The material underwent first a swelling process as indicated by the high amounts of water uptake upon incubation in the aqueous solution. This fact was expected since both materials, chitosan and starch are polysaccharides. Thus, they provide hydrophilic characteristics to the resulting microparticulated system. It could also be associated to the presence of α-amylase in the solution. Azevedo et al. reported that the partial degradation of the material resulting from enzymatic hydrolysis led to enhanced permeability[26] and thus increased values of water absorption over time. The action of the enzyme α-amylase on the degradation of the microparticles was clearly demonstrated. The material lost 67% of its weight after 60 days of incubation. Moreover, clear morphological changes could be detected. SEM microscopical observations revealed a complete loss of material integrity. Additionally, structural changes occur as result of the enzymatic action. This was evidenced by spectroscopic techniques such as FTIR and NMR. FTIR demonstrated the action of the α-amylase in cleaving the C—O—C glycosidic linkages of starch. Several authors have reported that any loss in starch content will result in a decrease in the absorbance in the carbohydrate fingerprint region (900–1200 cm^{-1}).[27,28] However, proton NMR showed the disappearance of several characteristic starch peaks after enzymatic action. A very pronounced effect was found on the α (1→4) glycosidic linkages of the starch. This was an expected result, since α-amylase is known to have a specific action on the mentioned starch bonds. This leads to the release of degradation products to the surrounding media, which could be measured. In the case of starch, glucose, and several other sugar molecules are expected to be released.[1,26] In fact, increasing amounts of reducing sugars were found in the supernatants of ST-CHT microparticles. The absence of specific α (1→4) glycosidic linkages in chitosan leads to the assumption that the amount of quantified sugars corresponds to the degradation of the starch material in the blend.

The impact of the enzymatic degradation over the release of GTM was observed ∼24 h after the immersion of the particles in the degradation media. The initial burst release (first 12 h) was not significantly changed. This result was to be expected if we consider that the initial burst release is attributable to the release of the drug that is present at the outermost layer of the microparticles and is thus quickly released before the enzymatic degradation starts to take place. Indeed, we have observed <5% weight loss after 12 h of microparticles' incubation. In our previous study, we have reported that about 54% of the entrapped drug was released from this material during the first 24 h.[11] When studying the drug release mechanism, a non-fickian diffusion was obtained for this microparticular system in PBS, thus indicating a contribution of polymer relaxation and drug diffusion factors to the GTM release. Here, the obtained results in the presence of the enzyme during the first 24 h are in accordance with the previous published data. At that time, the material has lost ∼15% of its initial weight and the porosity starts to be formed allowing the GTM to be easily released to the surrounding media. In fact, over 65% of the GTM was released 24 h after incubation of the microparticles, demonstrating the impact of the matrix biodegradation on the release of the entrapped drug. It can be concluded that after the initial burst release, a diffusion phenomenon is responsible for the release of the drug until enzymatic degradation starts to take place and finally controls the release mechanism.

Biodegradation is often desired when designing biomaterials for tissue engineering. This is especially true in drug delivery applications, were the main aim will be to the control of the delivery rate. However, there is major concern associated with the toxic effect of resulting degradation products and leachable molecules. Both unloaded and GTM-loaded ST-CHT particles developed here resulted biocompatible with the three cell types tested. A toxic effect was obtained, however, when GA was used during the particles' fabrication. This result is in accordance with previously published data on the toxicity of this crosslinking agent.[29] It is worth mentioning that this toxic effect was clearly concentration-dependent. Zhong et al. reported the use of vapors of GA to efficiently crosslink polysaccharide structures without toxic effect associated.[30] The use of TTP is also an alternative. As reported here, no toxic effect was observed even at high concentrations. However, the presence of starch clearly enhanced the biocompatibility of the ST-CHT blend. This polysaccharide has been reported as highly biocompatible. Its use as part of blended materials or composites will highly improve the biocompatibility of the resulting biomaterial.

CONCLUSION

The combination of chitosan and oxidized starch resulted in very hydrophilic and biodegradable microparticles. High values of water uptake (160%) and weight loss (67%) were found 60 days after enzymatic degradation *in vitro*. Additionally, increasing amounts of soluble sugars were released to the media. They resulted to be nontoxic when an extract of the particles (i.e., containing degradation products and leachable molecules) was added to cells in culture. Cell viability studies indicated that the ST-CHT microparticles had a negligible effect over SaOs-2 and L929 cell lines as well as AMSCs. Moreover, increasing amounts of starch in the blend composition resulted in increased cell proliferation. However, some toxicity was indeed observed for the microparticles produced using GA. A highly porous microparticular matrix was obtained after degradation. As a consequence, the matrix permeability also increased leading to increased water penetration. Thus, diffusion of the entrapped gentamicin was favored. The initial stage of drug release is then

controlled by diffusion. At a certain point, when the degradation process is taking place, the remaining amount of entrapped drug is released accordingly. Ultimately, the degradation rate of the material will control the release kinetics of the encapsulated drug.

The results obtained in this study allow us to corroborate starch as a biocompatible and biodegradable polysaccharide that can be used to enhance such features in its blended materials. We have been able to successfully conjugate chitosan with starch. Thereby, a highly biocompatible and biodegradable system was produced. This system could serve as a biomatrix not only for drug delivery, but also for many other tissue engineering and biomedical applications.

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